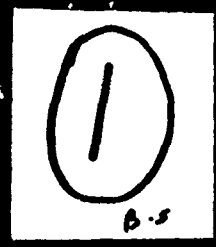
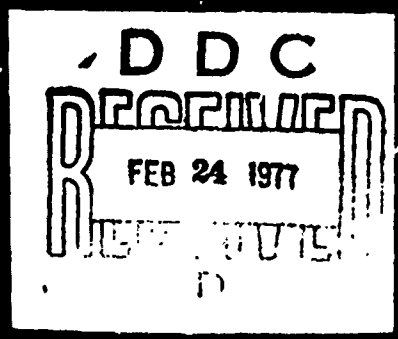
A METHOD FOR STUDYING CHANGES IN PROFIBRINOLYSIN TITER IN DOGS FOLLOWING IN VIVO INDUCTION OF FIBRINOLYTIC ACTIVITY

SCHOOL OF AVIATION MEDICINE
RANDOLPH AIR FORCE BASE, TEXAS

JANUARY 1959





A METHOD FOR STUDYING CHANGES IN PROFIBRINOLYSIN TITER IN DOGS FOLLOWING IN VIVO INDUCTION OF FIBRINOLYTIC ACTIVITY

D. R. CELANDER, Ph.D.
M. MASON GUEST, Ph.D.

Department of Biochemistry and Carter Physiology Laboratory
The University of Texas Medical Branch
Galveston, Texas

59-27

Air University
SCHOOL OF AVIATION MEDICINE, USAF
RANDOLPH AFB, TEXAS
January 1959

A METHOD FOR STUDYING CHANGES IN PROFIBRINOLYSIN TITER IN DOGS FOLLOWING IN VIVO INDUCTION OF FIBRINOLYTIC ACTIVITY

A two-stage assay for canine profibrinolysin (plasminogen) is described. This assay is suitable for following in vivo changes in profibrinolysin titer induced by various experimental procedures. Plasma profibrinolysin is concentrated by a low ionic strength, protamine sulfate precipitation technic and activated with staphylokinase. Diluted aliquots of the activated solution are then assayed for fibrinolytic activity. One unit is that amount of activity which at 37° C. and pH 7.25 will bring about lysis of 0.4 ml. of a 0.1 percent bovine fibrin clot in 300 seconds. The average dog plasma contains 19.4 units per milliliter. In general, increases in circulating fibrinolytic activity are associated with decreases in profibrinolysin.

Several problems are involved in the development of a rapid, convenient, and dependable method for estimation of canine profibrinolysin. First, the profibrinolysin must be separated from the plasma by a procedure which is quantitative and which will render it free of antifibrinolysin and other inhibitory agents. Second, a suitable activator must be available for the conversion of the profibrinolysin to fibrinolysin since it is only in the latter form that the activity of the enzyme can be measured. Finally, a substrate is necessary which will permit rapid and simple estimation of the proteolytic activity which develops in the presence of the kinase.

Methods for the isolation of the profibrinolysin fraction of plasma have depended on salt fractionation technics such as 30 percent saturation with ammonium sulfate (1, 2); 50 percent saturation with ammonium sulfate (3); alcohol fractionation as practiced by Richert (4); and a number of modifications (5, 6) of the low ionic strength, acid precipitation technic described by Milstone (7).

The conversion of profibrinolysin to fibrinolysin has been accomplished by triturition (8) and by treatment with such organic solvents as chloroform (1) and acid acetone (9) (neither procedure being suitable for analytic work), by incubation with kinases (that is, activators) of bacterial (7, 10, 11, 12), tissue (13), serum (14), and urinary (15-18) origin.

A solution of the substrate problem has been sought in fibrinogen (19-22), fibrin (1, 6, 7, 21, 23-26) (as many modifications of fibrin assay technics are available as there are investigators who have employed this substrate), powdered hide (15), unheated casein (5), heated casein (6, 27, 28), gelatin (11, 21, 29, 30), and synthetic substrates such as tosylarginine methyl ester (31).

As the result of technical or theoretical difficulties or both, no completely satisfactory assay for profibrinolysin has been available. We feel, however, that the procedure outlined below is satisfactory from both theoretic and technical aspects; it is in harmony with and takes full advantage of the information brought to light by experiments in this and other laboratories. It has been under test and in constant use since its development in 1953. The facts available to date have led us to conclude that, when properly carried out, it is a reliable procedure for the estimation of canine profibrinolysin.

METHODS AND MATERIALS

The 0.1 percent fibrin clot selected as the substrate in our test system is prepared from a 0.2 percent solution of bovine fibrinogen isolated by the freeze-thaw procedure of Ware

Received for publication on 4 September 1958.

et al. (32). The 0.2 percent solution is diluted with an equal volume of the lytic solution whose activity is to be measured and then clotted by stirring with a rod 2 mm. in diameter which has been dipped to a depth of 2 mm. into a solution of Parke-Davis Thrombin (2,500 units per ml. of 50 percent glycerol in saline). The clot forms within 8 to 10 seconds and is observed for lysis at 37° C. The end point is determined by the tilt-tube method described elsewhere (25).

The protamine sulfate used in the concentration of profibrinolysin from canine plasma is an 0.8 percent solution of Lilly salmine in 0.9 percent saline.

Staphylokinase, employed as an activator, is prepared from freshly isolated cultures of *Staphylococcus aureus*, as directed by Lewis and Ferguson (12). For use, the lyophilized powder is dissolved in saline to a final concentration of 4 to 8 mg. per milliliter.

Varidase® is made up in 0.9 percent saline so as to contain 15,000 units of streptokinase (23) per milliliter.

Imidazole buffer, pH 7.25 is prepared by dissolving 1.72 gm. of imidazole in 80 ml. of 0.1 normal HCl, adjusting to the desired pH, and diluting to 100 ml. (23).

EXPERIMENTAL

The assay of canine profibrinolysin is a two-stage procedure. In the first stage profibrinolysin, separated from interfering materials, is incubated with staphylokinase; in the second stage, the amount of fibrinolytic activity which develops is measured by determining the time of lysis of a standard fibrin clot formed in the presence of a diluted aliquot of the incubation mixture of stage 1.

Separation of profibrinolysin from plasma is accomplished as follows: nine volumes of canine blood mixed with one volume of 4 percent sodium citrate are centrifuged at 1,200 × g or more for 15 minutes. Two ml. of the supernatant plasma are withdrawn and mixed with 0.5 ml. of 0.8 percent protamine sulfate solution. The pH of the mixture is adjusted to 9.0 (glass electrode) and held at this pH at 28° C. for one hour. It is then lowered to 7.0 by adding 0.1 normal HCl dropwise and the plasma-protamine mixture is diluted to 40 ml. with distilled water. After allowing the tubes to stand for 15 minutes the precipitate which forms is collected by centrifugation at 1,500 × g for 10 minutes at 20° C. The supernatant which contains only traces of profibrinolysin is discarded. For best results, the precipitate should be used immediately, although experience has shown that it is possible to determine profibrinolysin on samples of the precipitate which have been chilled for 1 to 3 days or kept in the frozen state indefinitely.

To the precipitate at the bottom of the 50-ml. centrifuge tube in which it was deposited are added in order: 0.8 ml. of 0.9 percent saline, 0.9 ml. of imidazole buffer, pH 7.25, and 0.1 ml. of freshly thawed staphylokinase solution. The mixture is stirred and placed in a water bath at 28° C. This constitutes the first or activation stage of the assay. At 20 minutes, and at 15-minute intervals thereafter, up to 135 minutes, a 0.2-ml. aliquot of the incubation mixture is withdrawn and diluted with imidazole buffer to give lysis times ranging between 5 and 20 mintues when the diluted aliquots are incorporated into a standard fibrin clot.

The test clot is formed by mixing 0.2 ml. of the diluted aliquot with 0.2 ml. of 0.2 percent bovine fibrinogen and immediately clotting the mixture by the addition of excess thrombin on the end of a 2-mm. glass stirring rod. Dilution of the aliquots withdrawn from the incubation mixture is essential since at lysis times shorter than 5 minutes, clot formation is impaired. The incubation is continued until two successive aliquots withdrawn from the incubation mixture give the same lysis times at the same dilutions. With a satisfactory preparation of staphylokinase, maximum activation is usually attained within 90 to 120 minutes.

When the logarithm of the lysis time is plotted against the logarithm of concentration

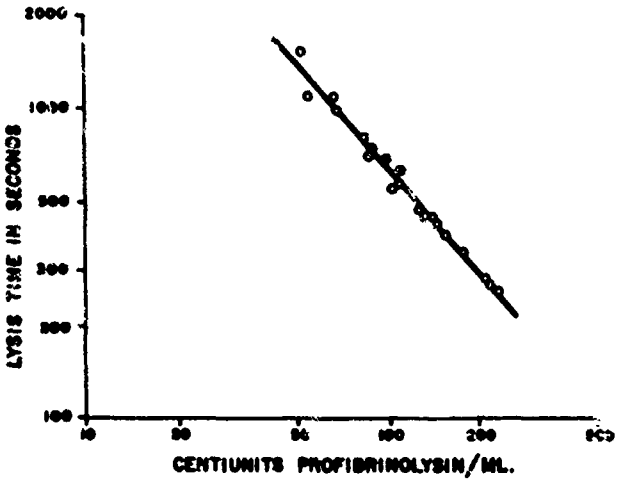


FIGURE 1.

Assay curve for canine profibrinolysin. See text for definition of unit and assay conditions. Curve has been corrected for 1:2 dilution of the test solution with bovine fibrinogen.

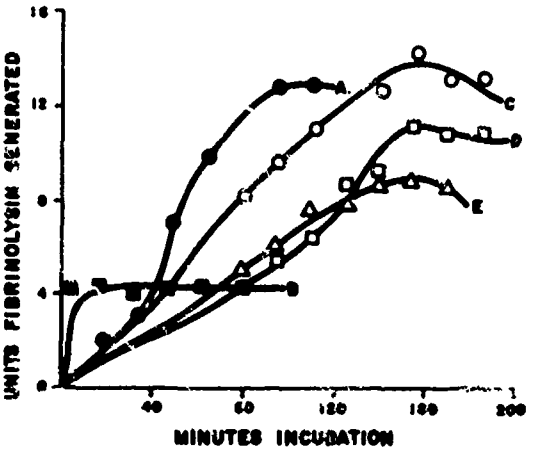


FIGURE 2.

Activation curves of canine profibrinolysin preparations isolated by various methods. Curves A and B are the activation curves of preparations obtained from the same plasma by the protamine sulfate technic (A) and isoelectric precipitation (B). Curves C, D, and E are profibrinolysin preparations isolated from a different plasma by the protamine sulfate technic (C), by alcohol precipitation (D), and by ammonium sulfate precipitation (E).

of profibrinolysin expressed in arbitrary units, a straight line relationship is obtained (fig. 1). The slope of this line is independent of the lot of staphylokinase used and of the degree of conversion of profibrinolysin to fibrinolysin. In this test, the unit is defined as that amount of fibrinolytic activity, measured at 37° C., which will completely lyse in 300 seconds 0.4 ml. of 0.1 percent fibrin clot in a system buffered at physiologic ionic strength with saline and imidazole at pH 7.25.

RESULTS

Figure 2, curve A depicts the development of fibrinolysin in the first stage of a two-stage procedure. It is apparent that the activation of canine profibrinolysin by the kinase of *Staphylococcus aureus* is not instantaneous, less than 40 percent of the total fibrinolytic activity having been generated in 20 minutes of incubation with that particular staphylokinase preparation. Aliquots of material isolated from the same plasma sample and incubated with aliquots of the same staphylokinase preparation will give curves of activation with identical shape. However, profibrinolysin preparations obtained from dog plasma before and after subjection of the animal to various experimental procedures or from the plasma of different dogs will not give identical curves when incubated with aliquots of the same preparation of staphylokinase; nor are identical curves obtained with aliquots prepared from the same plasma sample and treated with different lots of staphylokinase. Consequently, samples must be withdrawn from the incubation mixture so long as any increase in activity is obtained. The ultimate amount of fibrinolytic activity generated is independent of relatively wide variations in the potency of the staphylokinase preparation employed and constant for a given plasma sample.

Curve B of figure 2 was obtained by incubating the same amount of the same preparation of staphylokinase with the euglobulin prepared from plasma identical in volume and source to that used in curve A. Instead of using the protamine technic, the profibrinolysin-containing fraction was obtained by adjusting the pH of a 1:20 plasma dilution to 5.3 with 1 normal acetic acid. The shape of the curve is dissimilar and the total yield of fibrinolytic ac-

tivity lower than in curve A. The other curves of figure 2 represent activation of profibrinolysin-containing fractions obtained from another plasma by the protamine precipitation technic (curve C), by the alcohol precipitation technic of Richert et al. (4) (curve D), and by the ammonium sulfate precipitation technic of Lewis and Ferguson (2) (curve E).

Studies on recovery of canine profibrinolysin by the protamine isolation procedures were not carried out because canine profibrinolysin of sufficient purity for such purposes was not available. However, as indicated in figure 2, precipitates obtained with protamine yield higher activity than those obtained by technics involving alcohol, salt, or acid precipitation. The fact that no fibrinolysin could be detected in dialyzed supernatants which were incubated with staphylokinase for prolonged periods suggests that no profibrinolysin was present in such supernatants.

Figure 3 presents curves in which the profibrinolysin fractions were prepared from serum (curve A) and plasma (curve B) derived from the same blood sample. It can be seen that more activity develops and persists for a longer period of time when plasma is used.

When streptokinase (instead of staphylokinase) is used in stage 1, fibrinolytic activity develops more rapidly but does not achieve levels as high as those obtained with staphylokinase (fig. 4). Figure 5 presents a comparison of (1) a serially diluted, maximally staphylokinase-activated canine fibrinolysin; (2) a serially diluted, maximally streptokinase-activated canine fibrinolysin; (3) serially dilut-

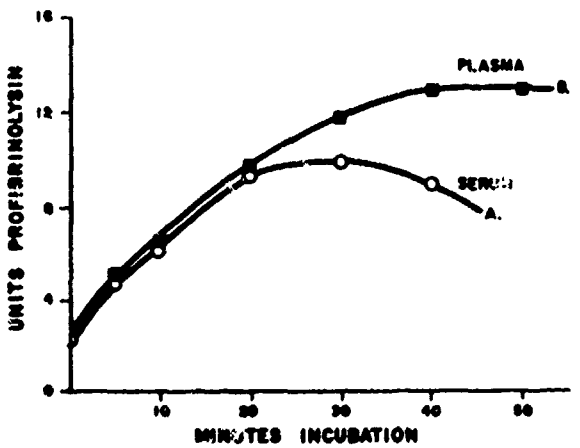


FIGURE 3.

Activation curves of canine profibrinolysin isolated (A) from plasma and (B) from serum. The plasma and serum were derived from the same whole blood sample.

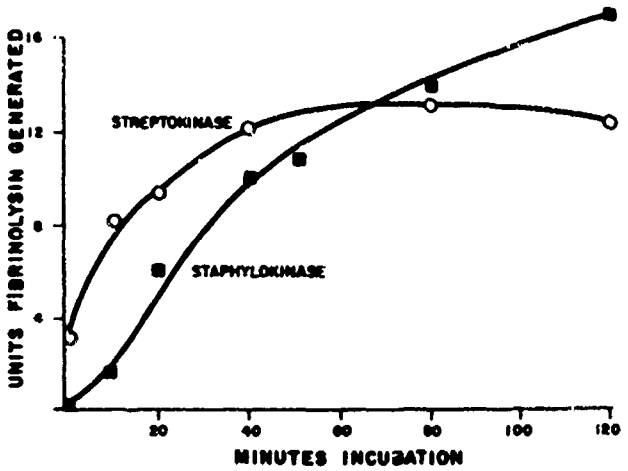


FIGURE 4.

Comparison of streptokinase and stapylokinase activation of canine profibrinolysin isolated by the protamine sulfate precipitation technic.

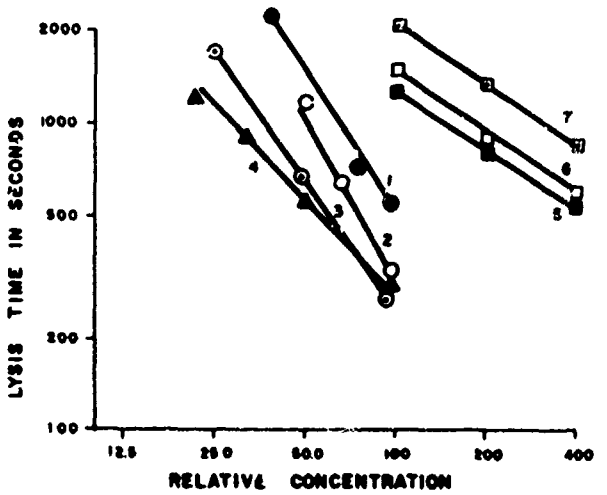


FIGURE 5.

Dilution curves of (1) staphylokinase-activated canine fibrinolysin; (2) streptokinase-activated canine fibrinolysin; (3) spontaneously active canine fibrinolysin; (4) trypsin; (5) streptokinase-activated canine plasma kinase; (6) streptokinase-activated human plasma kinase; and (7) urokinase.

ed spontaneously active canine fibrinolysin; (4) serially diluted trypsin; (5) serially diluted canine plasma, mixed with bovine fibrinogen and treated with streptokinase essentially as in the assay described for human plasma prokinase (34); (6) the assay curve for human prokinase; and (7) a plot of serially diluted urokinase. Curves 1, 2, 3, and 4 exhibit slopes which are strikingly similar and which reflect the dilution of a protease which acts directly upon fibrin. Curves 5, 6, and 7 denote the type of slope (much flatter) which is observed in a one-stage assay when one deals with lytic activity which develops as a result of the action of an agent whose primary effect is the conversion of bovine profibrinolysin present in the thrombin and fibrinogen reagents to fibrinolysin (35).

In figure 6 profibrinolysin titer is plotted along the abscissa in steps of 5 units and the number of animals falling into each unit category is plotted on the ordinate. Simple inspection of this histogram reveals that approximately half of the dogs have profibrinolysin titers which lie between 10 and 20 units. Quantitative examination of the data reveals that 67 percent of the animals have titers lying between 10 and 30 and that 95 percent of the animals exhibit profibrinolysin values lying between 0 and 40. The mean profibrinolysin titer in this group of 121 animals was 19.4 units; the standard error of the mean was 0.9. The variability observed is apparently real since the values are reproducible. It is important to mention in this connection that it was not possible to evaluate the history of each animal tested.

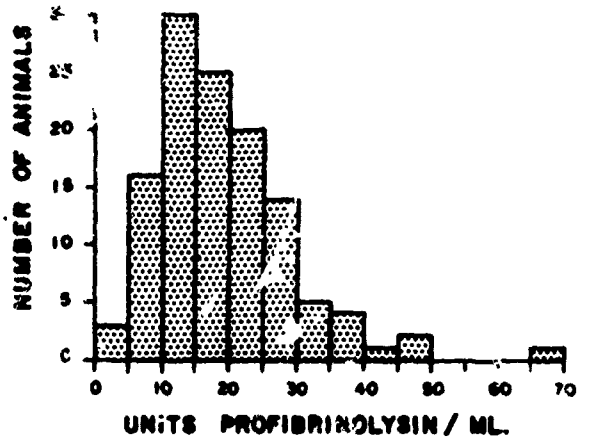


FIGURE 6.

Distribution of profibrinolysin titers in control dog plasmas.

Figure 7 illustrates the changes in profibrinolysin titer which occur following a number of experimental procedures which we have found will bring about the development of frank fibrinolytic activity. Frequently, even when frank fibrinolytic activity was not observed, more sensitive methods for detecting the presence of fibrinolysin revealed a unit correlation between fibrinolytic activity and decreases in profibrinolysin titer as measured by the technic described.

DISCUSSION

Comparison with the 5.3, low ionic strength technic as well as with methods involving alcohol and ammonium sulfate fractionation indicates that the protamine precipitation method yields canine profibrinolysin in a form which is relatively stable and which gives a high yield of fibrinolysin upon treatment with staphylokinase. The protamine sulfate precipitation technic employed here is similar to that used in the isolation of profibrinolysin from human plasma (25). The key difference lies in the fact that, in the dog, the yield appears to be more nearly quantitative and a larger proportion of the fibrinogen is precipitated. The fibrinogen provides a substrate which depresses the rate of destruction of the developing fibrinolysin. Tests have shown that shortly before maximum activity is achieved, the last traces of clottable material disappear from the incubation mixture. Thus maximum activity is measured at a time when the only fibrin present in the assay clot is the known amount added as bovine fibrinogen.

Comparison of staphylokinase with streptokinase as an activator reveals the superiority of staphylokinase in at least two respects: (1) greater total yields of fibrinolytic activity are obtained; and (2) canine plasma fractions treated with staphylokinase are not capable of con-

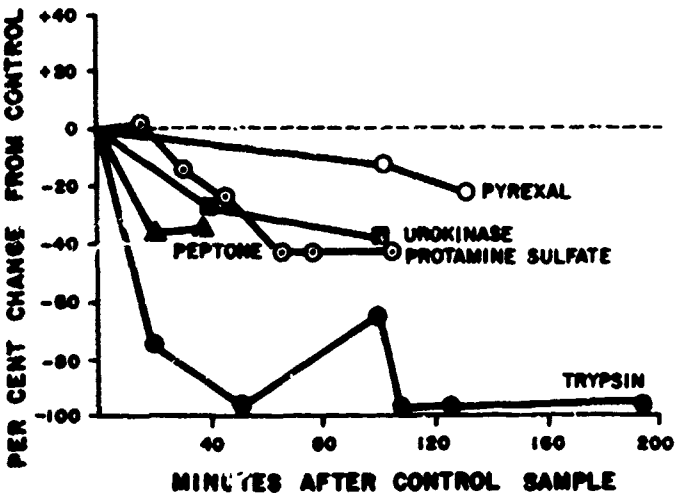


FIGURE 7.

Changes in profibrinolysin titers induced by the in vivo induction of fibrinolytic activity in dogs. Seven dogs received pyrexal®; 11, urokinase; 13, protamine sulfate; 18, peptone; and 29, trypsin. Each curve depicts the changes shown by one dog of an experimental group and is representative of the changes shown by other members of the group. Unless otherwise indicated the drugs were given by a single intravenous injection within 10 minutes after the withdrawal of the control blood sample. The amounts injected were as follows: pyrexal®, 0.15 mg./kg.; urokinase, 494 units/kg. (35); protamine sulfate, 16 mg./kg., given in divided doses of 4 mg./kg. at 5 minutes and 30 minutes and 8 mg./kg. at 60 minutes; peptone, 800 mg./kg.; trypsin, 7.5 mg./kg., in divided doses of 2.5 mg./kg. at 10 minutes and 5 mg./kg. at 105 minutes.

verting bovine profibrinolysin to fibrinolysin (fig. 4). Thus the second stage of the assay measures only the canine fibrinolysin generated even though there is some profibrinolysin present in the fibrinogen and thrombin used in the second stage. Streptokinase generates kinases in canine plasma fractions in much the same manner as in human plasma fractions (27, 34, 36). Since these activators can bring about the conversion of the profibrinolysin present in the bovine fibrinogen employed in the second stage of the assay, the use of streptokinase in such an assay results in false high values. It is important to re-emphasize then that the disadvantages which accrue through the use of profibrinolysin containing bovine reagents in assays involving streptokinase are absent from procedures in which staphylokinase is the activator. The curves presented in figure 4 indicate that the fibrinolysin which develops in the presence of staphylokinase dilutes in a manner identical to fibrinolysin which has been formed spontaneously. Both slopes are similar to that obtained with diluted trypsin, a protease, but are decidedly dissimilar to those obtained with dilution of urokinase, human plasma prokinase, and canine plasma prokinase. The slow decrease in activity observed with the one-stage bovine assay system which is typical for kinases, effecting lysis through conversion of bovine profibrinolysin to fibrinolysin, cannot be obtained with staphylokinase.

The opportunity of using native fibrinogen-fibrin systems afforded by staphylokinase possesses an additional advantage. The fibrinolysin is measured by causing a fibrin clot to develop in its presence, a situation which has

been shown to provide a most sensitive indicator of fibrinolytic activity (37).

Preparations of staphylokinase must be obtained from freshly isolated *Staphylococcus aureus*. Organisms which have been kept for long periods on slants or have been repeatedly subcultured are unsuitable since they form virtually no activator. Furthermore, strict adherence to the procedure originally outlined by Lewis and Ferguson (12) gives the most reproducible results.

The data presented in figure 7 show decreases in circulating profibrinolysin under a wide variety of experimental conditions which are known to induce fibrinolytic activity in vivo. The findings are entirely consistent with the surmise that fibrinolytic activity originates from the activation of circulating profibrinolysin and that as the former is increased, the latter is decreased.

SUMMARY

An assay method for canine profibrinolysin is outlined and unit activity defined. In this assay profibrinolysin, concentrated in a precipitate obtained by dilution of protamine sulfate-treated plasma, is activated with staphylokinase. The fibrinolytic activity generated is then measured by the time required for lysis of a standard bovine fibrin clot. The mean profibrinolysin titer of plasma samples from 121 control animals is 19.4 units. Increased fibrinolytic activity induced in vivo by various experimental procedures is found to be associated with decreased profibrinolysin titers.

REFERENCES

1. Loomis, E. C., C. George, Jr., and A. Ryder. Fibrinolysin: Nomenclature, unit, assay, preparation and properties. Arch. Biochem. 12:1-5 (1947).

2. Lewis, J. H., and J. H. Ferguson. Studies on a proteolytic enzyme system of the blood. II. Fibrinolysokinase activators for profibrinolysin. J. Clin. Invest. 29:1059-1068 (1950).

3. Soicher, R. R., P. Richards, and B. Rose. The estimation of profibrinolysin. J. Lab. & Clin. Med. 43:495-500 (1954).

4. Richert, D. A. Proteolytic activity of hemophilic plasma. Proc. Soc. Exp. Biol. & Med. 70-743-745 (1949).

5. Downie, G. R., and E. E. Cliffton. A method for assay of serum proteolytic activity. Proc. Soc. Exp. Biol. & Med. 71:138-141 (1949).

6. Remmert, L. F., and P. P. Cohen. Partial purification and properties of a proteolytic enzyme of human serum. J. Biol. Chem. 181:431-448 (1949).

7. Milstone, H. A factor in normal human blood which participates in streptococcal fibrinolysis. J. Immunol. 42:109-116 (1941).

8. Fischer, A. Mechanism of the proteolytic activity of malignant tissue cells. Nature 157:442 (1946).

9. Von Kaulla, K. N. Extraction of fibrinolytic enzyme from the blood. Nature 164:408 (1949).

10. Tillett, W. S., and R. L. Garner. The fibrinolytic activity of hemolytic streptococci. J. Exper. Med. 58:485-502 (1933).

11. Christensen, L. R. Streptococcal fibrinolysis: a proteolytic reaction due to a serum enzyme activated by streptococcal fibrinolysin. J. Gen. Physiol. 28:363-383 (1945).

12. Lewis, J. H., and J. H. Ferguson. A proteolytic enzyme system of the blood. III. Activation of dog serum profibrinolysin by staphylokinase. Am. J. Physiol. 166:594-602 (1951).

13. Permin, P. M. Properties of the fibrinokinase-fibrinolysin system. Nature. 160:571-572 (1947).

14. Lewis, J. H., and J. H. Ferguson. Studies on a proteolytic enzyme system of the blood. IV. Activation of profibrinolysin by serum fibrinolysokinase. Proc. Soc. Exp. Biol. & Med. 78:184-188 (1951).

15. Williams, J. R. B. The fibrinolytic activity of urine. Brit. J. Exper. Path. 32:530-537 (1951).

16. Astrup, T., and I. Sterndorff. An activator of plasminogen in normal urine. Proc. Soc. Exp. Biol. & Med. 81:675-678 (1952).

17. Sobel, G. W., S. R. Mohler, N. W. Jones, A. B. C. Dowdy, and M. M. Guest. Urokinase: an activator of plasma profibrinolysin extracted from urine. Am. J. Physiol. 171:768-769 (1952).

18. Mohler, S. R., D. R. Celander, and M. M. Guest. Distribution of urokinase among the common mammals. School of Aviation Medicine, USAF, Report No. 57-147, Sept. 1957. Am. J. Physiol. 192:186-190 (1958).

19. Ferguson, J. H. Assay of tryptases by lysis of fibrinogen. Proc. Soc. Exp. Biol. & Med. 52:243-245 (1943).

20. Ungar, G., and S. H. Mist. Release of serum fibrinolysin by specific antigen, peptone and certain polysaccharides. J. Exper. Med. 90-39-51 (1949).

21. Kaplan, M. H., H. J. Tagnon, C. S. Davidson, and F. H. L. Taylor. Studies on blood coagulation: The nature and properties of a proteolytic enzyme derived from plasma. J. Clin. Invest. 21:533-537 (1942).

22. Geiger, W. B. Protease activation in immune reactions. J. Immunol. 68:11-18 (1952).

23. Christensen, L. R. Methods for measuring the activity of components of the streptococcal fibrinolytic system and streptococcal desoxyribonuclease. J. Clin. Invest. 28:163-172 (1949).

24. Guest, M. M., A. G. Ware, and W. H. Seegers. A quantitative study of antifibrinolysin in chick plasma: Increase in antifibrinolysin activity during pteroylglutamic acid deficiency. Am. J. Physiol. 150:661-669 (1947).

25. Guest, M. M. Profibrinolysin, antifibrinolysin, fibrinogen and urine fibrinolytic factors in the human subject. J. Clin. Invest. 33:1553-1559 (1954).

26. Lassen, M. Heat denaturation of plasminogen in the fibrin plate method. Acta physiol. scandinav. 27:371 (1952).

27. Mullertz, S. Formation and properties of the activator of plasminogen and of human and bovine plasmin. Biochem. J. 61:424-434 (1956).

28. Warner, R. C., and E. Polis. On the presence of a proteolytic enzyme in casein. J. Am. Chem. Soc. 67:529-532 (1945).

29. Lundblad, G. Investigations on plasmin. I. On the proteolytic activity of plasmin. Acta chem. scandinav. 3:354-358 (1949).

30. Ungar, G., and E. Damgaard. Studies on the fibrinolysin-antifibrinolysin system in serum. I. Action of the anterior pituitary, adrenal cortex and spleen. J. Exper. Med. 93:89-97 (1950).

31. Troll, W., S. Sherry, and J. Wachman. The action of plasmin on synthetic substrates. J. Biol. Chem. 208:85-93 (1954).

32. Ware, A. G., M. M. Guest, and W. H. Seegers. Fibrinogen: With special reference to its preparation and certain properties of the product. Arch. Biochem. 13:231-236 (1947).

33. Mertz, E. T., and C. A. Owen. Imidazole buffer: its use in blood clotting studies. Proc. Soc. Exper. Biol. & Med. 43:204-206 (1940).

34. Guest, M. M., and D. R. Celander. Assay of human plasma prokinase. Am. J. Physiol. 187:602-603 (1956).

35. Celander, D. R., R. P. Langlinais, and M. M. Guest. The application of foam technique to the partial purification of a urine activator of plasma profibrinolysin. Arch. Biochem. 55:286-287 (1955).

36. Mullertz, S., and M. Lassen. An activator system in blood indispensable for formation of plasmin by streptokinase. Proc. Soc. Exper. Biol. & Med. 82:264-268 (1953).

37. Celander, D. R., and M. M. Guest. Factors influencing susceptibility of fibrin and fibrinogen to proteolysis by fibrinolysin. School of Aviation Medicine, USAF, Report No. 56-48, May 1956. Arch. Biochem. 72:176-188 (1957).